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DOI: <https://doi.org/10.1016/j.jhep.2011.04.014>

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ZORA URL: <https://doi.org/10.5167/uzh-52584>

Journal Article

Accepted Version

Originally published at:

Guyot, C; Stieger, B (2011). Interaction of bile salts with rat canalicular membrane vesicles: Evidence for bile salt resistant microdomains. *Journal of Hepatology*, 55(6):1368-1376.

DOI: <https://doi.org/10.1016/j.jhep.2011.04.014>

**Interaction of bile salts with rat canalicular membrane vesicles: Evidence
for bile salt resistant microdomains**

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Word count: 4996

Number of figures and tables: 9 figures in the main text, 2 tables in the supplementary material.

Grant support:

This work was supported by Grants #31003A-112524 and #31003A-124652 from the Swiss National Science Foundation and by Grant #07C75 from Novartis Foundation for Biomedical Research (Basel, Switzerland).

Abbreviations: BS, bile salts; ABC, ATP binding cassette; Bsep, bile salt export pump; Mdr, multidrug resistance protein; cLPM, canalicular liver plasma membrane; BSRM, bile salt resistant microdomain; CMC, critical micellar concentration; APN, aminopeptidase N; DPPIV, dipeptidylpeptidase IV; Mrp2, multidrug resistance-associated protein 2.

Abstract:

Background & Aims: Canalicular phosphatidylcholine and cholesterol secretion requires the coordinate action of the ATP binding cassette transporters: the bile salt export pump (Bsep) for bile salts (BS) and the phosphatidylcholine translocator multidrug resistance protein 2 (Mdr2). After their secretion, phosphatidylcholine and BS form mixed micelles acting as acceptors for canalicular cholesterol. We have shown that the canalicular liver plasma membrane (cLPM) contains lipid rafts enriched in sphingomyelin and cholesterol. As BS have detergent properties and their concentration in the canaliculus is very high, we tested the hypothesis that the canalicular membrane contains BS resistant microdomains.

Methods: Isolated cLPMs were extracted at 4°C with different BS or detergents and subjected to flotation in sucrose step gradients followed by Western blotting and lipid composition analysis.

Results: Incubating cLPMs with increasing taurocholate concentrations revealed the presence of BS resistant microdomains. These microdomains were found with different BS in the presence and absence of lipids and contained the rafts markers reggie-1/-2 and caveolin-1 and canalicular transporters Bsep, Mrp2 and Abcg5, the later independent of the presence of lipids. BS resistant microdomains contain mainly cholesterol, phosphatidylcholine and phosphatidylethanolamine. Extraction of cLPMs with a mixture of different BS similar to rat bile revealed a comparable microdomain composition.

Conclusions: cLPM contains BS resistant microdomains potentially protecting the cLPM against the detergent action of BS. Combination of different BS has no synergistic effect on microdomain composition.

Key words: Liver, canalicular plasma membrane, lipid composition, microdomains.

Introduction:

Bile is mainly composed of bile salts (BS), lipids and organic anions. BS and biliary lipids form mixed micelles [1]. These mixed micelles can act as acceptors for poorly water soluble compounds, e.g. cholesterol. Canalicular lipid secretion needs three members of the ATP-binding cassette (ABC) superfamily of transporters: the bile salt export pump Bsep (Abcb11), the phosphatidylcholine translocator Mdr2 (Abcb4) and the heterodimeric transporter Abcg5/Abcg8 [2,3]. Biliary lipid secretion has been investigated in inherited human liver disease and in genetically modified animals. The combined findings indicate that after secretion by Bsep into the canaliculus, BS extract phosphatidylcholine from the outer leaflet and solubilise it in mixed micelles. The supply of phosphatidylcholine to the outer leaflet of the canalicular liver plasma membrane (cLPM) is maintained by Mdr2. The mixed micelles act as acceptors for cholesterol, whereby Abcg5/Abcg8 helps to release cholesterol from the outer leaflet of the cLPM [2]. The following observations support this concept: 1.) Patients with inherited defects of BSEP develop progressive familial intrahepatic cholestasis type 2 and have practically no primary BS and no lipids in bile [4] 2.) Mice lacking functional Mdr2 do not secrete phosphatidylcholine into bile, even when challenged by high bile salt secretion and display minimal cholesterol secretion [5]. 3.) Mice with disrupted genes for Abcg5 or Abcg8 have markedly reduced biliary cholesterol secretion [6].

While it is now well established that biliary lipid secretion depends on the correct interplay of canalicular ABC-transporters, there is also evidence that biophysical processes are involved in biliary lipid secretion. For example, if livers of mice with a disrupted *Mdr2* gene are challenged with a high bile salt load, cholesterol secretion is induced [5]. Furthermore, retrograde infusion of BS into the bile duct releases a burst of phospholipids from the liver (presumably from the canaliculus) into bile [7]. Finally, *in vitro* incubation of cLPM vesicles with unconjugated and conjugated BS leads to a preferential release of phosphatidylcholine, even though it amounts to only 35% of total canalicular phospholipids [8,9]. This *in vitro*

interaction of BS with plasma membranes is not restricted to cLPM, but has also been observed with rat kidney brush border membrane vesicles and with erythrocytes from different species [9,10]. Also, conjugated BS are weakly toxic and unconjugated bile acids are more strongly toxic to bacteria [11,12]. Hence, it is evident that the cLPM needs to have special biophysical properties and/or protective mechanisms, which prevent it from being solubilised by the high concentrations of BS found in the canaliculus.

One such protective factor could be cholesterol, which preferentially associates with sphingomyelin, a sphingolipid found in the outer leaflet of membranes [13,14]. Cholesterol decreases the extent of bile salt induced phospholipids solubilisation from membranes [15,16]. Phosphatidylcholine release from membranes correlates inversely with their sphingomyelin content, whereby the cLPM presents an exception [9]. Sphingomyelin and cholesterol are enriched in detergent resistant membrane microdomains or lipid rafts, where they are tightly packed in a liquid ordered state [17,18].

We have recently demonstrated that the cLPM contains two types of detergent resistant microdomains differing in their protein and lipid composition [19]. Triton X-100 microdomains contain reggie-1/flotillin-2 and reggie-2/flotillin-1 as well as sphingomyelin, but very little cholesterol. In contrast, Lubrol WX microdomains are enriched in caveolin-1, cholesterol and sphingomyelin. Furthermore, Lubrol WX microdomains contain phospholipids as well as the ABC-transporters Bsep, Abcg5 and Mdr2. Hence, Lubrol WX microdomains contain the machinery for canalicular bile formation and could represent the starting place for canalicular lipid secretion [19]. Consequently, we here tested this hypothesis by treating cLPMs with BS, which are naturally occurring detergents produced by hepatocytes.

Materials and Methods:

Chemicals and methods describing immunoblotting and lipids analysis are given in the online supporting material.

Animals: Male Sprague-Dawley rats (180-200g) obtained from Harlan (Horst, Netherlands) received humane care in accordance with local and federal regulations and were kept under standard conditions. The animal experiments were approved by the local supervisory board on animal experimentation.

Model bile composition: Based on literature canalicular bile was modeled as follows: 10mM β -taumuricholic acid, 2mM α -taumuricholic acid, 9mM taurocholic acid, 2.5mM taurochenodeoxycholic acid, 1.5mM taurodeoxycholic acid or 25mM taurocholate, 0.7mM Cholesterol, 5mM phosphatidylcholine (supplementary table1).

Isolation of bile salt resistant microdomains (BSRMs) from cLPMs: cLPMs were isolated as described [20] and stored in liquid nitrogen. To isolate BSRMs, 1 mg cLPMs were thawed on ice and diluted in TNE (150mM NaCl, 1mM EDTA, 20mM Tris-HCl pH 7.4) containing antipain and leupeptin 1 μ g/ml each and 1mM PMSF. The suspension was mixed with twofold concentrated BS or detergent in TNE. After extraction of cLPMs with BS or detergents, centrifugation and work up were performed as described in [19]. Each experiment was performed at least twice independently.

Results:

In order to test whether BS are able to trigger the formation of BSRMs, cLPMs were treated with taurocholate close to its critical micellar concentration (CMC) (supplementary table2). After extraction with 8mM taurocholate and flotation through a sucrose gradient, BSRMs were found in the upper part of the gradient (fractions 4-6). As demonstrated in Fig.1, these BSRMs contain 16% of the total protein recovered in the gradient and 72% of the protein content was found with the solubilised material at the bottom of the gradient. Analysis of the

fractions by Western blotting for raft markers identified more than 70% of caveolin-1, reggie-1/flotillin-2 and reggie-2/flotillin-1 in the BSRMs (Fig.2).

To test the efficiency of bile salt induced cLPMs extraction at 8mM taurocholate, we next used concentrations above and below the CMC. Protein distribution after treatment with 12mM taurocholate showed barely any difference to 8mM treatment, demonstrating complete extraction close to the CMC (Fig.1). In contrast, extraction with 4mM taurocholate yielded 67% of the total protein content in BSRMs reflecting an incomplete extraction (Fig.1). Interestingly, well below the CMC a portion of the cLPMs was solubilised as proteins were recovered from fractions 9 to 12 representing the loading part of the gradient. With only about 5% of the input recovered in the soluble part of the gradient, extraction of raft markers was poor after treatment with 4mM taurocholate (Fig.2). We analysed all fractions for canalicular markers proteins and selected canalicular ABC-transporters. Actin was analysed for determining a potential interaction of microdomains with the cytoskeleton. Treatment with 4mM taurocholate incompletely solubilised canalicular markers since no soluble APN was detectable and only 15% of DPPIV was found in the soluble fractions. cLPMs treatment with 8mM or 12mM taurocholate increased the amount of APN and DPPIV in the soluble part of the gradient to about 20% and 40%, respectively. 4mM taurocholate incompletely solubilised the canalicular ABC-transporters Bsep, multidrug resistance-associated protein 2 (Mrp2), Abcg5 and Mdr1 with 8%, 25%, 8% and 1%, respectively residing in the soluble part of the gradient. Treatment with 8mM and 12mM taurocholate resulted in about 50% of Bsep and Mrp2, 60% of Abcg5 and 40% of Mdr1 in the soluble part of the gradient. cLPMs treatment with 4mM taurocholate yielded 75% actin in BSRMs, while 8mM or 12mM taurocholate rendered actin mainly soluble with only small amount remaining associated with BSRMs (Fig.2). Therefore, actin was not further analysed.

Based on these results, additional BS were used at concentrations close to their CMCs (supplementary table2). Three BS with increasing relative hydrophobicity:

tauroursodeoxycholate, glycocholate and taurodeoxycholate were investigated. After extraction with 3mM tauroursodeoxycholate, 69% of the total protein content remains in BSRMs and only 26% were solubilised. Therefore, we used also 10mM tauroursodeoxycholate, which is markedly above CMC and found that protein solubilisation was increased (50% of total protein were soluble) and only 37% of the total protein content was found in BSRMs (Fig.3). Interestingly, regardless of the different solubilisation efficiency of the total protein, the distribution pattern of rafts markers, canalicular markers and ABC-transporters was comparable (Fig.4). In both conditions the major part of the membrane proteins (transporters and canalicular markers) remains in BSRMs and the solubilised protein remains below 12% of total protein content. Extraction with 12mM glycocholate and 3mM taurodeoxycholate gave similar protein distribution of 9% and 11% in BSRMs and 89% and 86% in the soluble part of the gradient, respectively (Fig.3) resembling the protein distribution after extraction with 8mM or 12mM taurocholate (Fig.1). The raft markers, caveolin-1 and reggie-1/flotillin-2 and reggie-2/flotillin-1, resided mainly in BSRMs after treatment with 12mM glycocholate or 3mM taurodeoxycholate (more than 65%, Fig.4). 27% of APN and 36% of DPPIV were soluble at 12mM glycocholate while 47% of APN and 51% of DPPIV were soluble at 3mM taurodeoxycholate suggesting minor differences in the solubilisation efficiency of BS. After treatment with 12mM glycocholate or 3mM taurodeoxycholate, ABC-transporters Bsep and Abcg5 were mainly soluble since only about 15% was in BSRMs, Mrp2 was distributed equally in BSRMs and in the soluble part of the gradient and a small amount (less than 4%) pelleted. Mdr1 was also equally distributed between BSRMs and the soluble part after 3mM taurodeoxycholate treatment and after 12mM glycocholate treatment 60% of Mdr1 was in BSRMs and 35% was soluble (Fig.4).

While different BS show different maximum secretory rates [21] no major differences in BSRMs composition induced by different BS were observed. We therefore next tested the non-micelle forming bile salt taurodehydrocholate [22] as negative control and the bile salt

derived detergent, CHAPS (CMC 6mM, [23]) as positive control. Octylglucoside as a microdomain destabilizing detergent was included as a third control (CMC 25mM, [23]). cLPMs extraction with 12mM taurodehydrocholate solubilised 31% of the total protein while 58% remained in BSRMs (Fig.5). All studied membrane proteins were mainly found in the BSRMs (>70%) and less than 10% was soluble, excepted for Mrp2, of which 50% were in BSRMs, 24% solubilised and 4% in the pellet (Fig.6). 6mM CHAPS induced the formation of some detergent resistant membranes with 17% of protein floating (Fig.5) and 75% soluble demonstrating that it is an additional mild detergent inducing microdomains, which contain rafts markers, canalicular markers and 33% of Bsep, 31% of Mrp2, 8% of Abcg5 and 77% of Mdr1.

Extraction with 25mM octylglucoside solubilised 71% of total protein and shifted 27% into the floating part. Increasing octylglucoside concentration to 50mM (2x CMC) lead to almost complete solubilisation of cLPMs with 99% of total protein remaining in the soluble gradient part (Fig.5). Extraction with 25mM octylglucoside barely solubilised raft markers or canalicular proteins since more than 80% recovered in BSRMs. ABC-transporters were equally distributed between the soluble part and BSRMs except for Mdr1, which remained with 88% in BSRMs (Fig.6). After treatment with 50mM octylglucoside rafts markers are still partly floating: 37% and 32% for caveolin-1 and reggie-2/flotillin-1 and 59% for reggie-1/flotillin-2, with a sizeable part becoming soluble: 40% of caveolin-1, 35% of reggie-1/flotillin-2 and 55% of reggie-2/flotillin-1 (Fig.6). Canalicular markers APN and DPPIV were solubilised to about 90% and ABC-transporters with the exception of Mrp2 (7% floating) were almost completely soluble (Fig.6).

Since bile contains different BS we extracted cLPMs with a modeled bile (Fig.7A,B). Compared to individual BS, the transport protein composition of the BSRMs was indistinguishable, while less total protein (2%) segregates into BSRMs, suggesting a more efficient extraction. Of the proteins, less of Bsep (7%) and of Abcg5 (5%) partitioned into

BSRMs. Finally, we extracted cLPMs with 25mM TC in the presence and absence of cholesterol and phosphatidylcholine (fig.7C-F). Using TC for the extraction, no differences to model bile were observed. After extraction with a mixture of TC and lipids, the distribution of the transport proteins and of reggie-2/flotillin1 was comparable to the control while reggie-1/flotillin-2 and caveolin-1 were recovered almost quantitatively in the BSRMs.

Next, we analyzed the cholesterol content in the fractions after extraction with different BS and detergents. Fig.8 shows that cholesterol almost quantitatively partitioned into BSRMs after extraction with 12mM taurodehydrocholate (87%), 3 and 10mM tauroursodeoxycholate (82% and 93%, respectively) and 4mM taurocholate (90%). In contrast, after treatment with 8mM and 12mM taurocholate and 12mM glycocholate less cholesterol was found in BSRMs: 59%, 61% and 47%, respectively, which is in the range after treatment with model bile (not shown). In addition cholesterol was recovered in the soluble portion of the gradient (17%, 13% and 19%, respectively) and a small amount was found in the pellet (8%, 10% and 13%, respectively). After treatment with 3mM taurodeoxycholate, 78% of the cholesterol was found in the BSRMs, 5% was solubilised and less than 1% was found in the pellet. Cholesterol distribution after treatment with CHAPS and 25mM octylglucoside showed a similar pattern, with most cholesterol in the insoluble part and 2% and 5%, respectively in the soluble part. After treatment with 50mM octylglucoside 35% of the cholesterol was insoluble and 52% was soluble (Fig.8).

Finally, phospholipids distribution was investigated. Fig.9 demonstrates that after treatment with 4mM taurocholate phospholipids were only detectable in BSRMs, confirming incomplete extraction (see Fig.1). Increasing the concentration to 8mM or 12mM taurocholate (as well as model bile, not shown) did not markedly change the phospholipids composition of BSRMs. Interestingly phosphatidylcholine (and to a much lesser extent phosphatidylethanolamine) was recovered in the soluble part of the gradient (Fig.9B,C),

confirming the preferential release of phosphatidylcholine from cLPM after incubation with taurocholate [9].

Discussion:

This study extends our previous findings on detergent resistant microdomains in cLPM [19]. We demonstrate here that different BS can induce microdomains in cLPMs. These microdomains contain the raft markers caveolin-1 and reggie-1/flotillin-2 and reggie-2/flotillin-1 as well as part of the canalicular ABC-transporters Bsep, Mrp2, Abcg5 and Mdr1 associated with specific canalicular lipids. Hence, BSRMs resemble the previously described Lubrol WX microdomains [19]. Using a modeled bile (25mM BS, fig.7) had no major impact on BSRM protein composition, but reduced total protein content. This is reproduced after extractions with 25mM of taurocholate in the presence or absence of lipids at concentrations found in bile. This indicates that the BS concentration used was not limiting for inducing BSRMs. It further supports the concept that lipids in bile predominantly protect the biliary tree [24]. The marked reduction of total protein content of BSRMs by model bile and 25mM taurocholate can be explained by removal of loosely adhering or trapped proteins. This assumption is supported by the finding that extraction of cLPMs with 1M NaSCN releases up to 40% of total protein (Guyot and Stieger unpublished). The different behaviour of caveolin-1 in the presence and absence of lipids may reflect its partitioning into different types of microdomains [19].

Canalicular BS and lipid secretion is a complex process involving coordinate actions of several transporters and necessitating a specific lipid composition of the cLPM. This specific lipid composition protects the cLPM from the detergent action of the high BS concentration in the canaliculus, which may exceed with 50mM in hepatic bile [25] the CMCs of BS (supplementary table2). Secreted BS extract phosphatidylcholine from the outer leaflet of the cLPM and quickly form mixed micelles, which have a protective effect against the toxic

effect of BS on the luminal membranes of the biliary tree [24]. Whether mixed micelles are formed fast enough in the canaliculus to protect cLPM remains open. Our comparison of extraction with taurocholate in the presence and absence of lipids suggests that the specific composition of cLPM protects it from solubilisation by canalicular BS even in the absence of mixed micelles. If however cLPMs are challenged by high BS loads from the cytoplasmic side by infusing rats with different BS at very high BS loads the cLPM is perturbed, which leads to a decrease of the BS secretory rate [21]. The secretory maximum is highest for tauroursodeoxycholate and correlates inversely with BS hydrophobicity. The reduction of BS secretion has been attributed to the toxic effects of BS towards the cLPM, but not to mere saturation of the BS efflux system(s) [21]. Indeed, characterization of rat Bsep in Sf9 cell membrane vesicles showed comparable intrinsic clearances for different BS [26]. Several observations support the concept of a protective mechanism in the cLPM: First, retrograde infusion of BS releases phosphatidylcholine and cholesterol in a dose dependent manner into bile, while infusion of the non-micelle forming BS taurodehydrocholate does practically not liberate lipids, and infusion of the BS-derived detergent CHAPS non-specifically extracts phospholipids [7]. Furthermore, the amount of phospholipids released by taurocholate infusion is not linearly related to the concentration of the infused BS. Combined with our data, these findings [7] suggest that microdomains present in the cLPM act as protective element. Second, the fatty acid composition of phosphatidylcholine in cLPM and in bile differs, requiring a process selecting distinct phosphatidylcholine for biliary secretion [27]. Third, comparison of sphingomyelin species in cLPM with sphingomyelin in bile provides evidence for laterally separated domains in the cLPM [28]. Such a lateral selection process could be based on microdomains and depend on intracanalicular concentrations and hydrophobicities of secreted BS. Fourth, cholesterol is a critical component of lipid microdomains. Feeding mice lacking Atp8b1 with a cholate diet renders them cholestatic and markedly reduces the cholesterol to phospholipid ratio in cLPM [29]. These and other authors

have shown that the activity of Bsep depends on membrane cholesterol content [29,30]. Atp8b1 likely is an aminophospholipid flippase keeping the aminophospholipid concentration in the outer leaflet of the cLPM low [31]. Hence, altered aminophospholipid gradients across the cLPM result in a partial loss of cholesterol from cLPM. BSRMs show a relative enrichment of PE in microdomains (Fig.9). This relative enrichment may be an additional element of stabilization of BSRMs against BS induced lipid release into the canaliculus.

Partitioning of canalicular transporters into BSRMs could add an additional protective element against the detergent action of BS. BSRMs contain the transporters required for canalicular bile formation and are enriched in cholesterol. The function of the ABC-transporters is modulated by membrane cholesterol, since a positive correlation between membrane cholesterol content and transporter activity for Bsep [30], BCRP [32] and MRP2 (Guyot and Stieger unpublished) expressed in Sf9 cells and for Bsep in cLPM [29] has been demonstrated. Stimulation of Mrp2 will increase BS independent bile flow and consequently lower BS concentration in the canaliculus. A choleretic stimulus associates aquaporin 8 with rafts in the cLPM [33]. Interestingly, stimulation of BS independent bile flow by bosentan activation of Mrp2 in the presence of ongoing BS secretion reduces canalicular phospholipids and cholesterol secretion [34]. This further supports the notion that intracanalicular BS concentration has to be in a narrow range for allowing undisturbed canalicular lipid secretion [35].

Comparing the protein composition of BSRMs with Lubrol WX microdomains [19] reveals a similar composition. Both contain the raft markers caveolin-1, reggie-1/flotillin-2 and reggie-2/flotillin-1 as well as ABC-transporters and cholesterol. Immunolocalisation of caveolin-1 [19] and Bsep [9] revealed their expression in canalicular microvilli. It is conceivable that BSRMs and Lubrol WX microdomains resemble the same entities. The raft markers sphingomyelin and cholesterol partition into Lubrol WX and BSRMs. However, both lipids

also partition into Triton X-100 microdomains [19,33]. It remains therefore open, whether these two lipids simultaneously partition into both types of microdomains in cLPM.

The observation that octylglucoside, even at high concentration, extracts rafts markers and hence does not completely solubilise microdomains (Fig.6) is unexpected. Octylglucoside is recommended for complete solubilisation of membranes including microdomains [36], but has also been reported to not completely solubilise membranes [37]. This discrepancy may relate to technical differences, as octylglucoside is frequently used in pelleting conditions. As cholesterol-sphingolipid complexes are needed to protect the canaliculus against the detergent action of BS, they can be expected also in microdomains in the planar, connecting membranes between microvilli, which are devoid of Bsep and Mrp2 [19]. This arrangement of different lipids into specific microdomains points to a dual role of lipids in physiology of bile formation. First, in the cLPM, lipids by forming microdomains may both stabilize the bilayer and indirectly regulate canalicular BS and lipid secretion. Second, in bile, lipids are predominantly protecting the biliary tree from the toxic action of BS [24].

In conclusion, we presented evidence for BSRMs in the cLPM containing key constituents of canalicular BS and lipid secretion. Due to their specific lipid composition, these microdomains may be involved in the regulation of the activity of key transporters involved in bile formation.

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Figure Legends:

Figure 1: Total protein distribution after extraction with increasing taurocholate (TC) concentrations. cLPMs were extracted with TC and floated on discontinuous sucrose gradients, twelve 1mL fractions were collected from the top of the gradient, the pellet was resuspended in 1mL 5% sucrose. Fractions were analysed for protein content. Low-density fractions 4-6 contain BSRMs; high-density fractions 9-12 contain solubilised proteins.

Figure 2: Distribution of rafts markers, canalicular markers and ABC-transporters after extraction with increasing TC concentrations. cLPMs were treated as in fig.1. Fractions were analyzed Western blotting for raft markers caveolin-1, reggie-1/flotillin-2 and reggie-2/flotillin-1; for canalicular markers APN and DPPIV; for actin as cytoskeletal marker and for the ABC-transporters Bsep, Abcg5, Mrp2 and Mdr1. Lower panels show densitometric analysis of Western blots (grey bars: BSRMs, black bars: soluble, open bars pellet).

Figure 3: Total protein distribution after cLPMs extraction with tauroursodeoxycholate (TUDC), glycocholate (GC), taurodeoxycholate (TDC). cLPMs were extracted with 3 and 10mM TUDC, 12mM GC or 3mM TDC and floated on discontinuous sucrose gradients. Fractions were analysed for protein content.

Figure 4: Distribution of rafts markers, canalicular markers and ABC-transporters after extraction with TUDC, GC and TDC. cLPMs were extracted with 3 and 10mM TUDC, 12mM GC or 3mM TDC and floated on discontinuous sucrose gradients. Fractions were analyzed by Western blotting for raft and canalicular markers proteins and ABC-transporters, followed by densitometric analysis (grey bars: BSRMs, black bars: soluble, open bars pellet).

Figure 5: Total protein distribution after cLPMs extraction with taurodehydrocholate (TDHC), CHAPS and octylglucoside (OG). cLPMs were extracted with 12mM TDHC, 6mM CHAPS or 25 and 50mM of OG. Fractions were analysed for protein content.

Figure 6: Distribution of rafts markers, canalicular markers and ABC-transporters after extraction with TDHC, CHAPS or OG. cLPMs were extracted with 12mM TDHC, 6mM CHAPS or 25 and 50mM of OG. Fractions were analyzed by Western blotting for raft and canalicular markers proteins and ABC-transporters, followed by densitometric analysis (grey bars: BSRMs, black bars: soluble, open bars pellet).

Figure 7: Distribution of total protein, rafts markers, canalicular markers and ABC-transporters distribution after extraction with model bile (A,B), 25mM TC (C,D) or 25mM TC (E,F) and lipids. cLPMs were extracted as described in Materials and Methods. Fractions were analysed for total protein content (A,C,E) and analyzed by Western blotting for raft and canalicular markers proteins and ABC-transporters (B,D,F), followed by densitometric analysis (grey bars: BSRMs, black bars: soluble, open bars pellet).

Figure 8: Cholesterol distribution after extraction with BS and detergents. cLPMs were treated with different BS or detergents. Fractions were subjected to lipid extraction and analysed for cholesterol content, which is expressed as percent of total cholesterol content (grey bars: BSRMs, black bars: soluble, open bars pellet).

Figure 9: Phospholipids distribution after extraction with taurocholate.

cLPMs were treated with 4mM (A), 8mM (B) or 12mM (C) taurocholate. Fractions were subjected to lipid extraction and analysed for phospholipids content. Individual phospholipids were compared to a standard mixture of sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), cardiolipin (CL) and phosphatidylethanolamine (PE). a-c: increasing amounts of standard, 1-12: Fractions, P: pellet, M1 and M2 lipids extracted from untreated cLPMs.

Figure 1

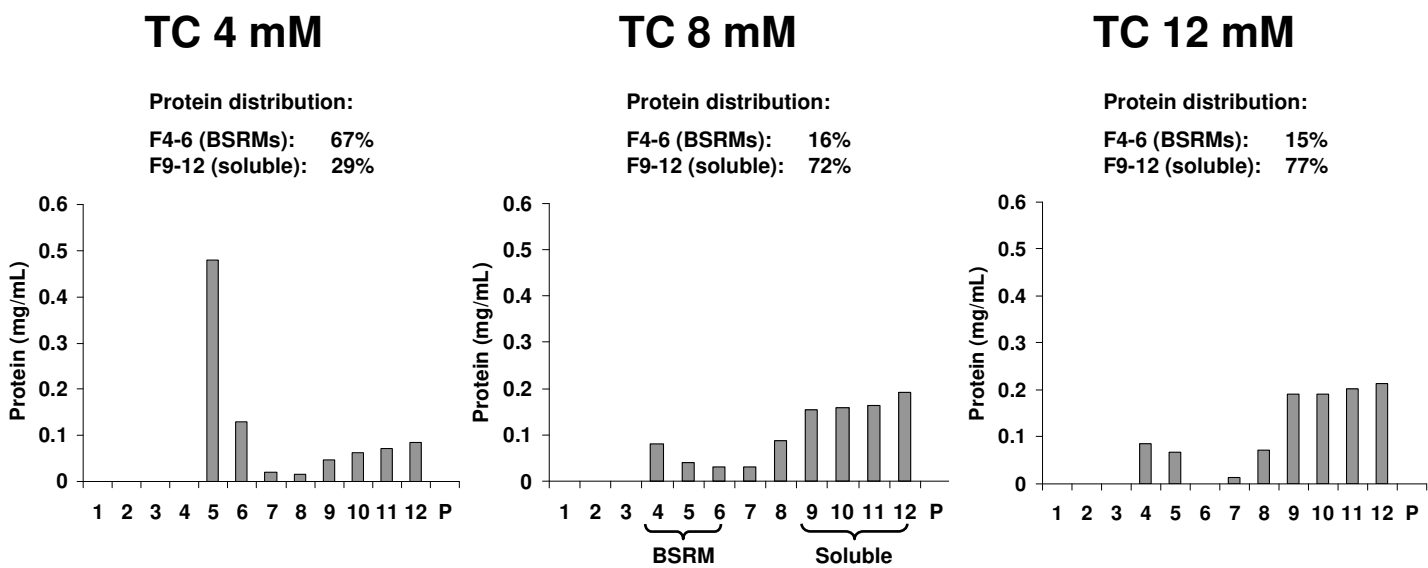


Figure 2

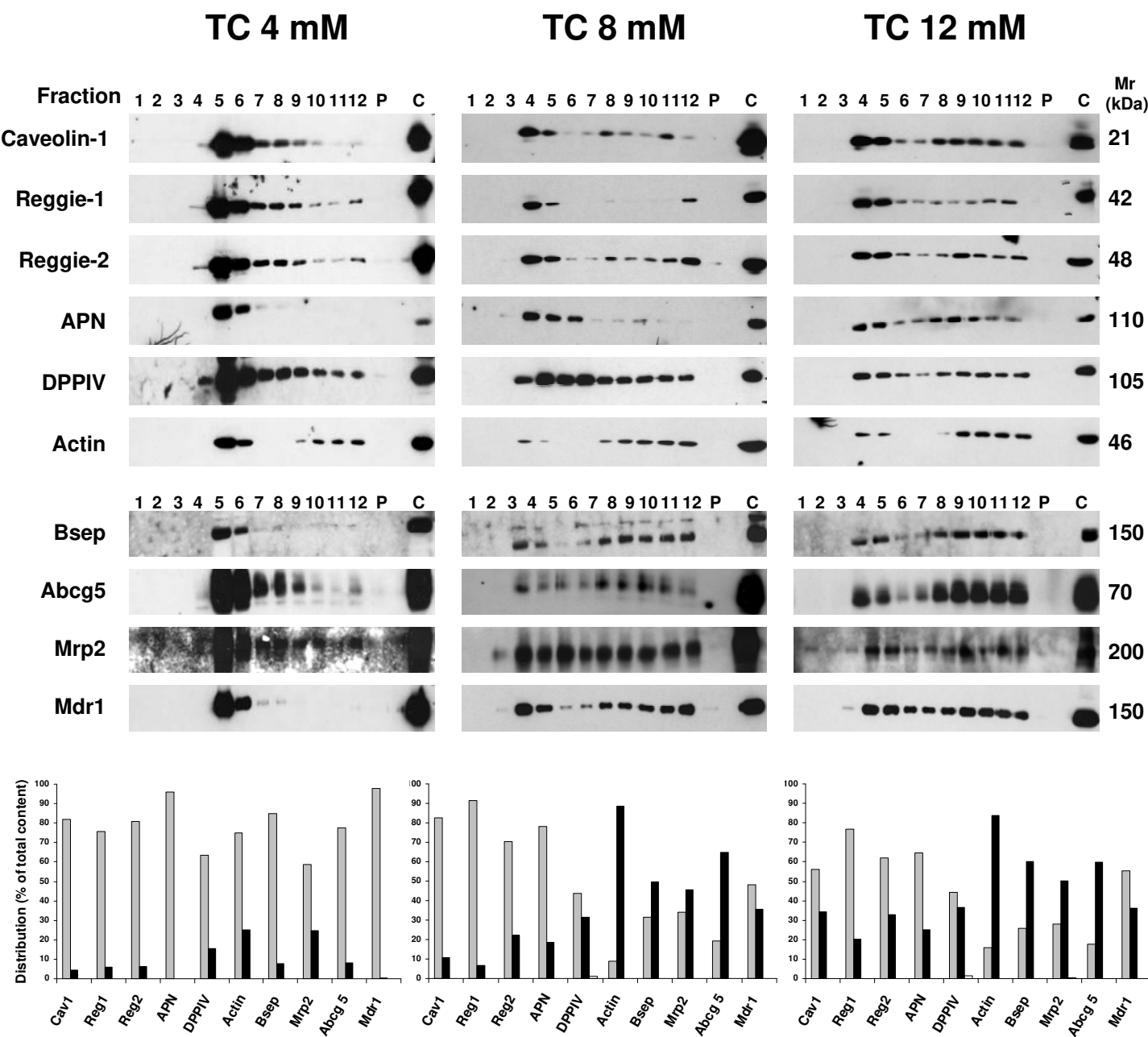


Figure 3

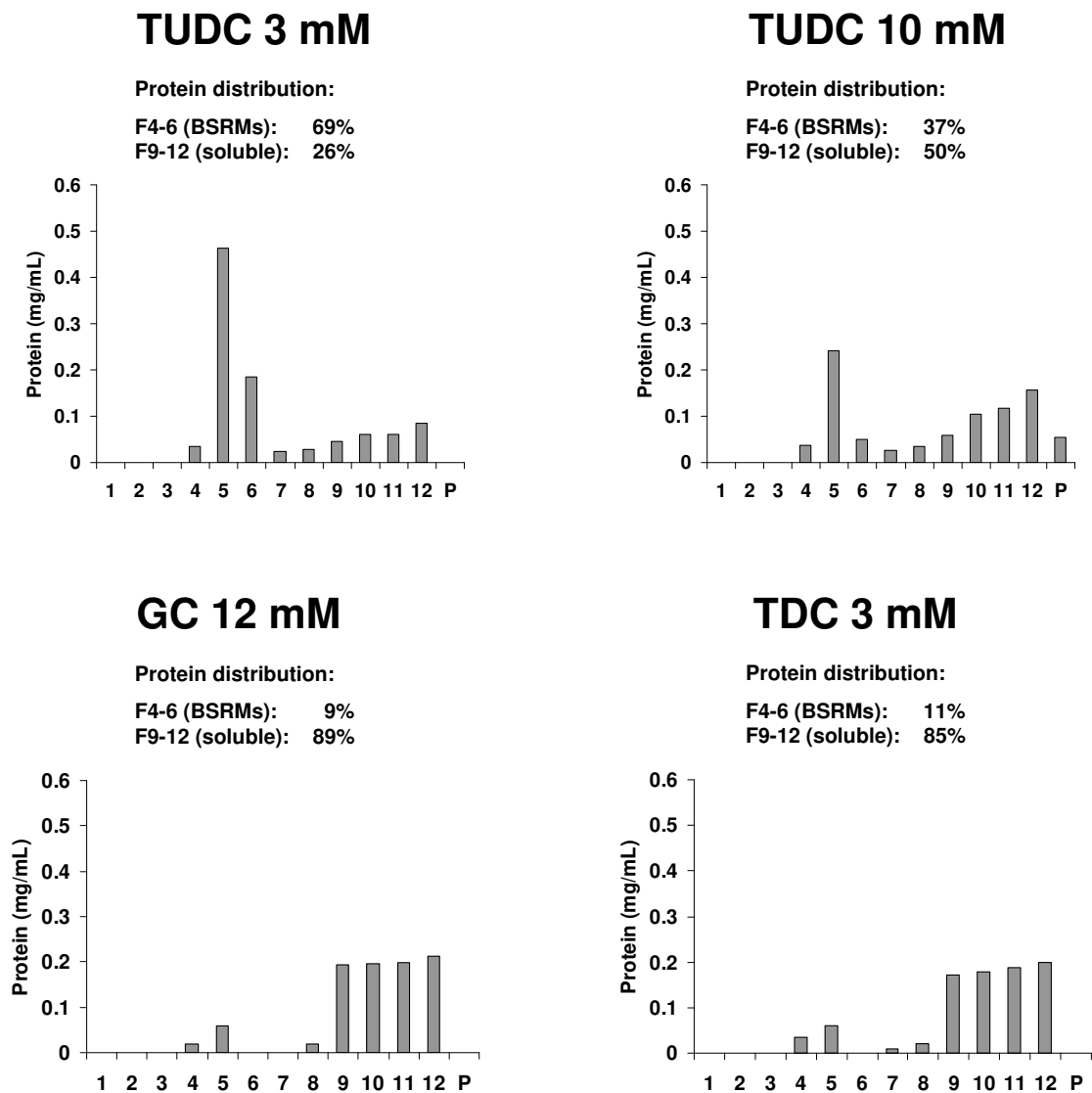


Figure 4

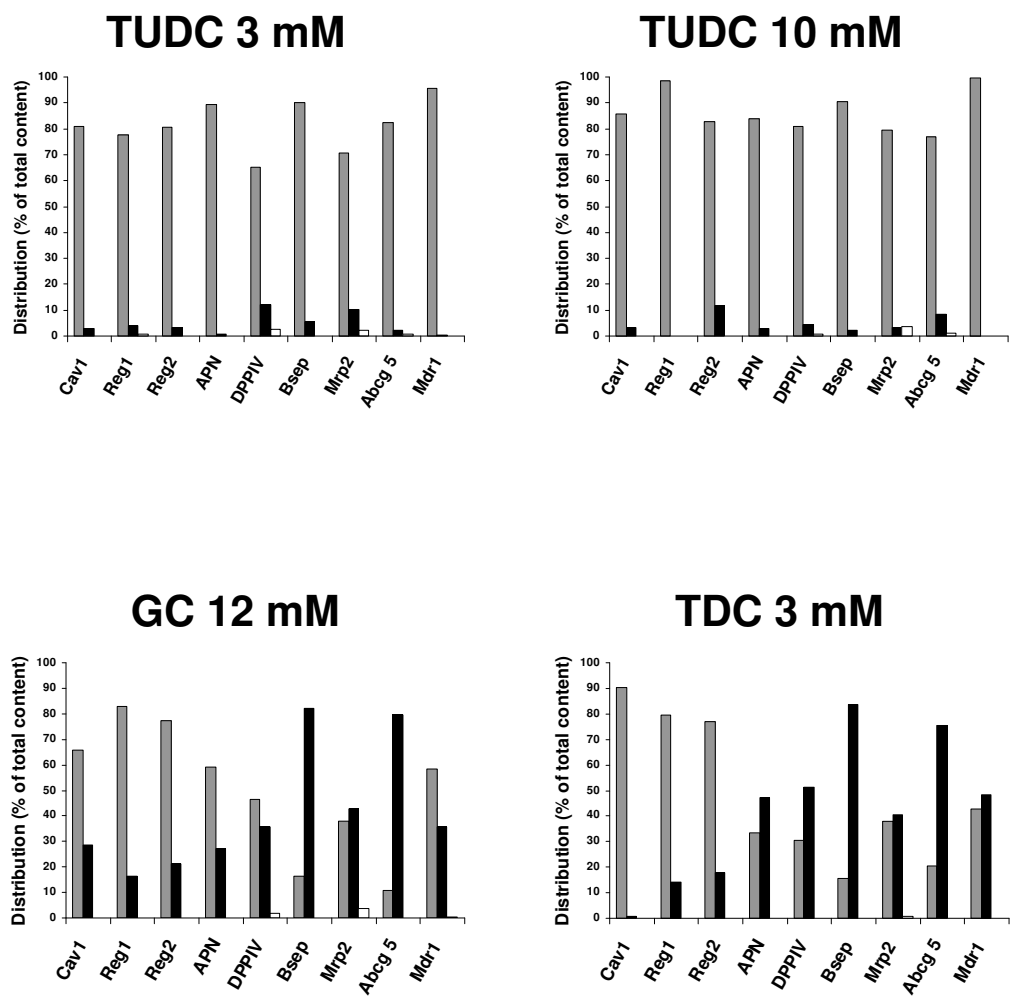


Figure 5

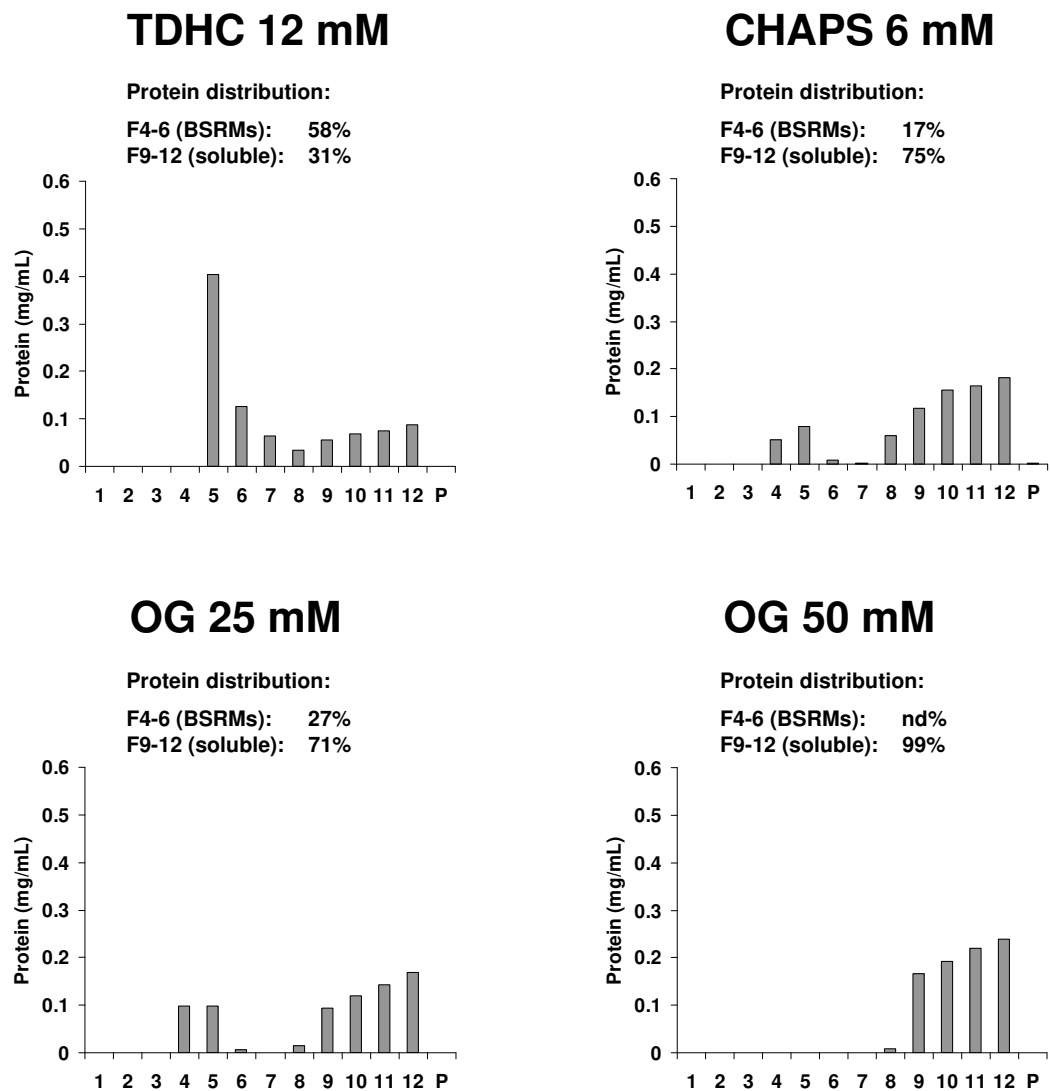


Figure 6

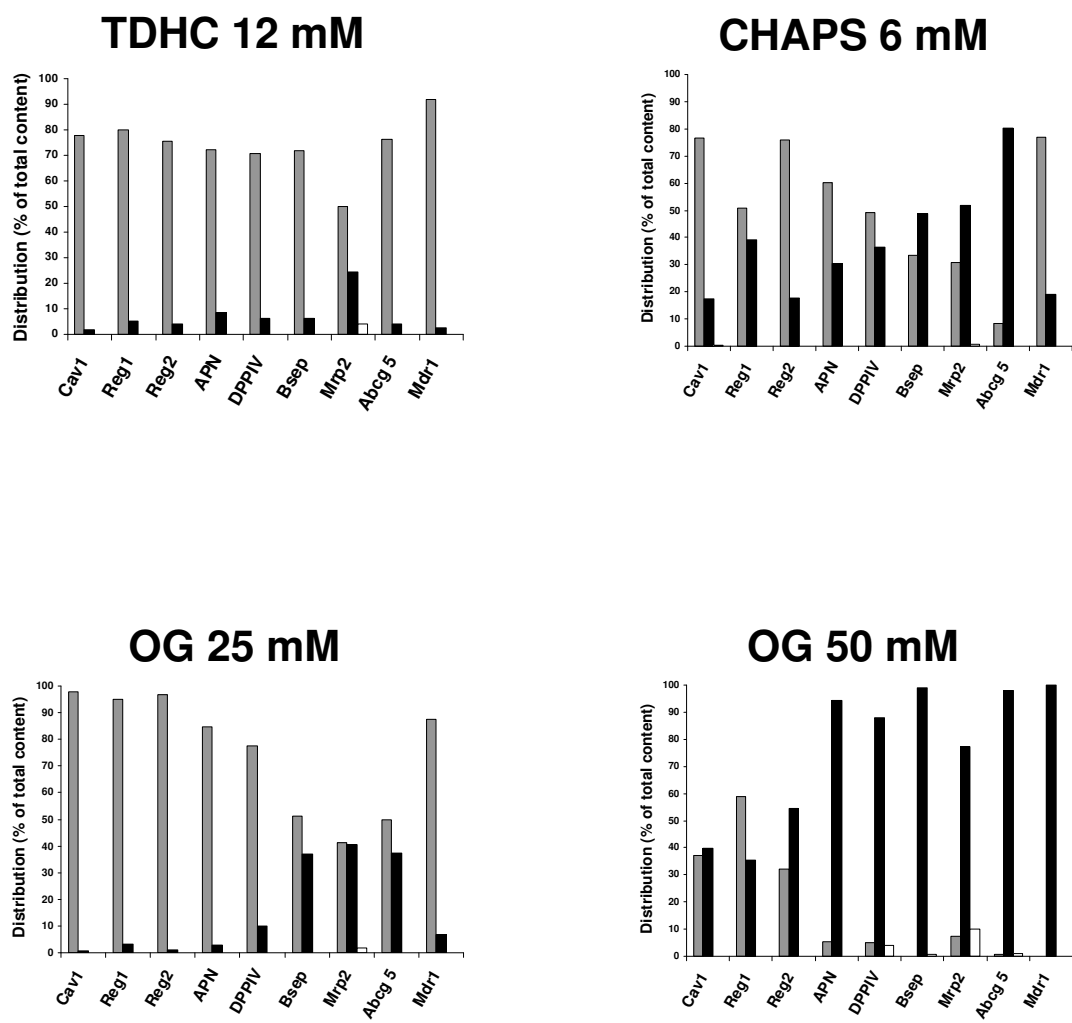


Figure 7

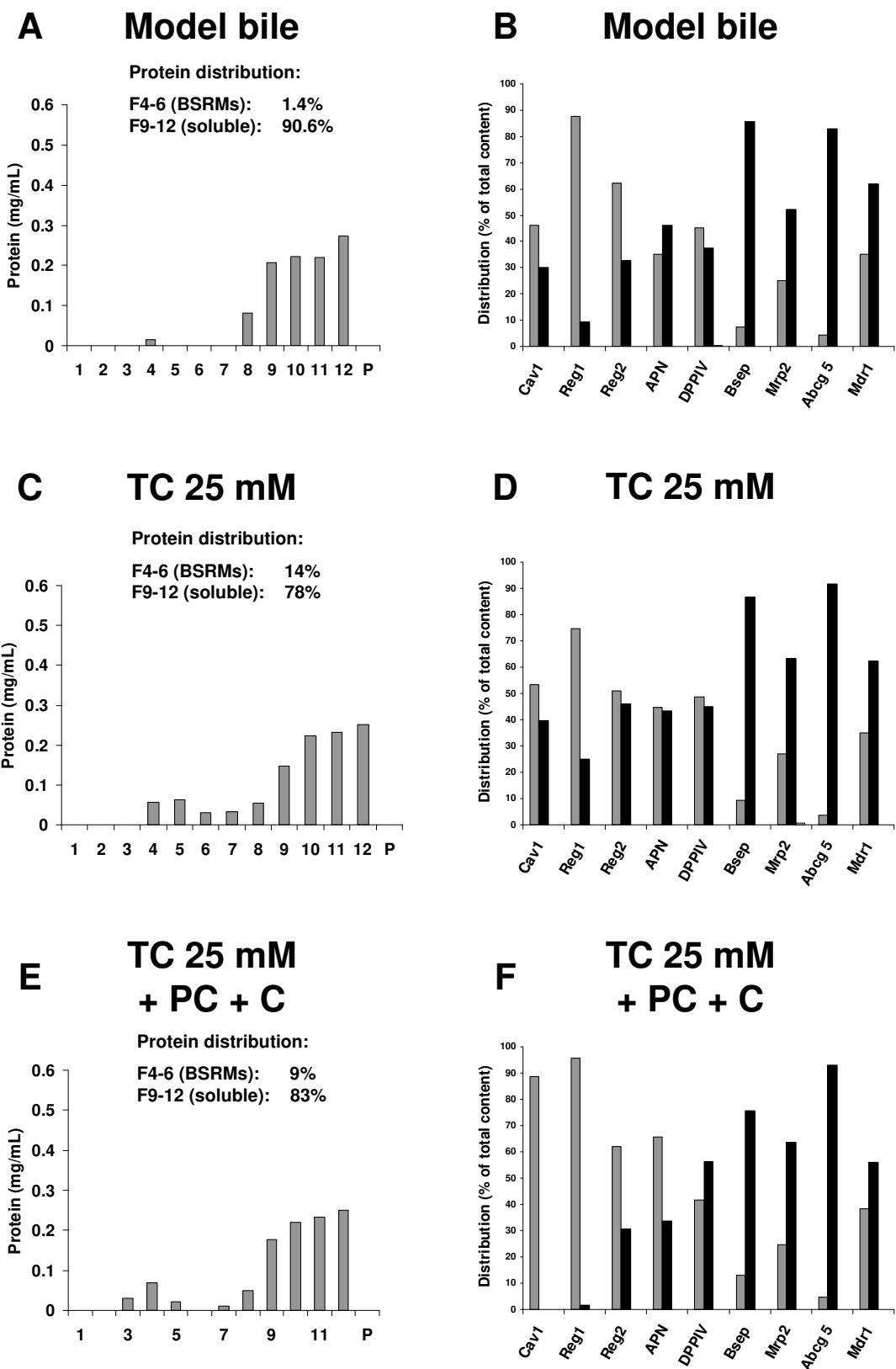


Figure 8

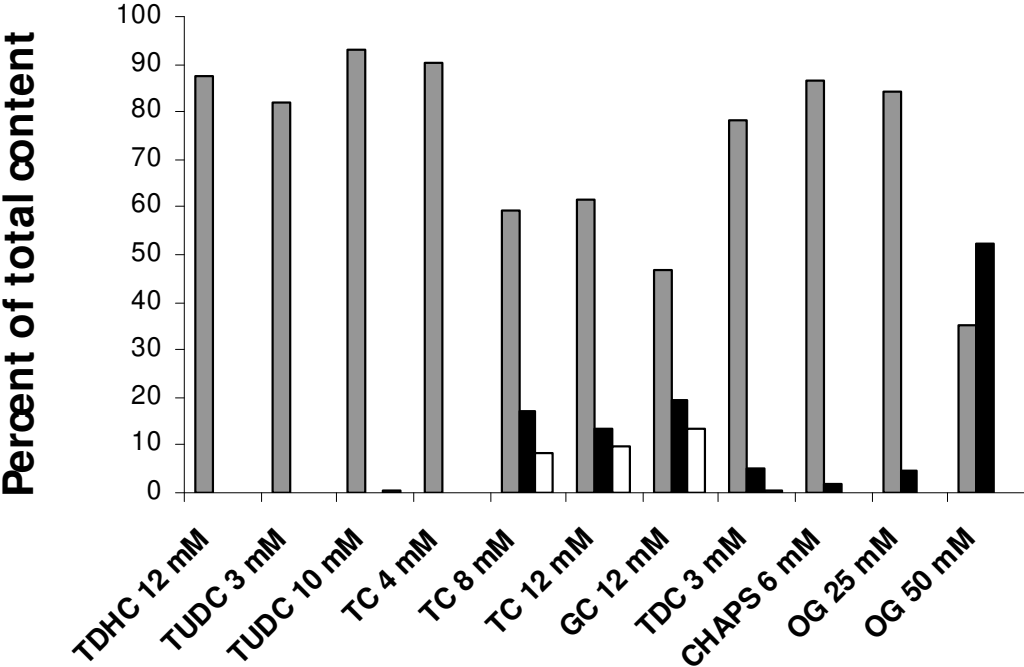
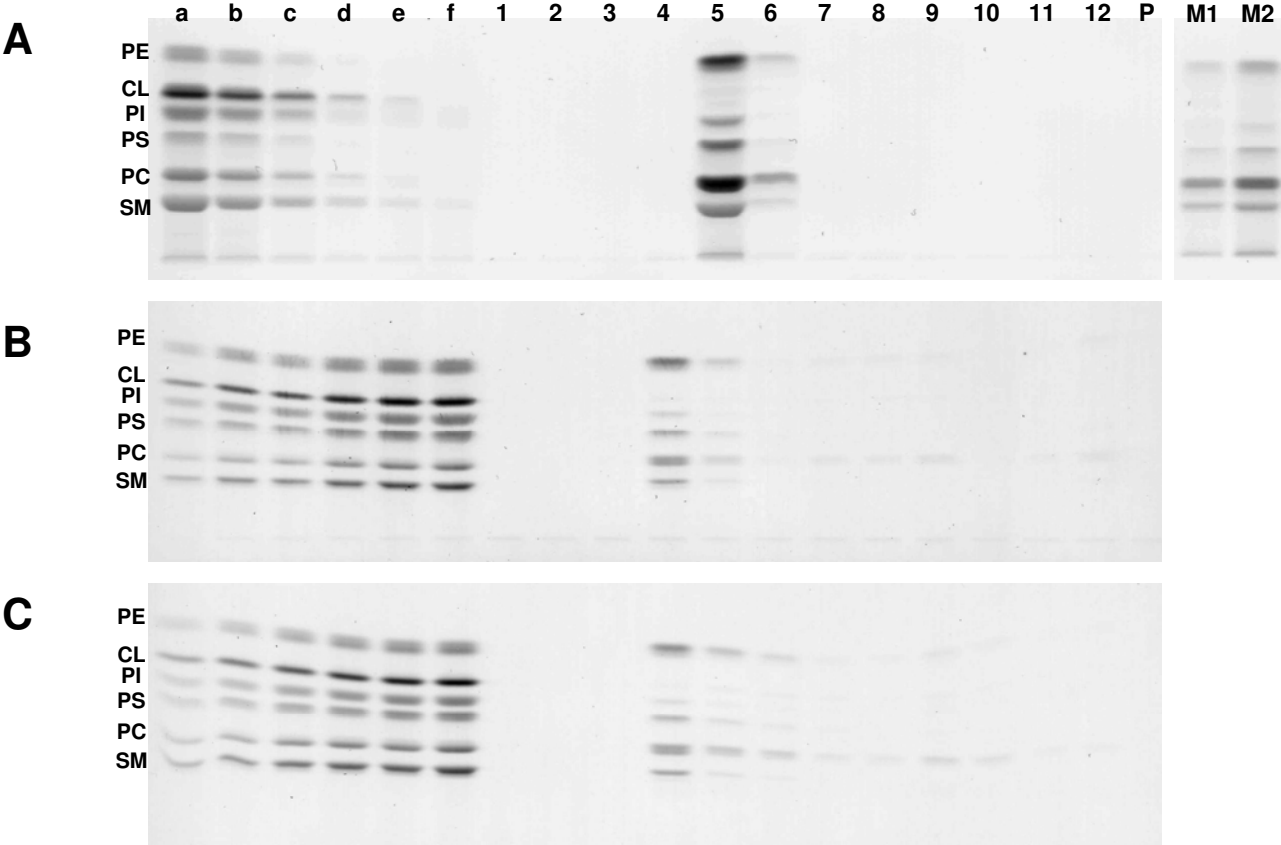


Figure 9



Supplementary material:

Chemicals: Phospholipids standards, phosphatidylcholine, cholesterol and bile salts were purchased from Sigma-Aldrich (St. Louis, MO), except taurodehydrocholate which was from Calbiochem (La Jolla, CA), CHAPS was from Biorad (Richmond, CA), octylglucoside from Bachem (Bubendorf, Switzerland), alpha and beta muricholic acid from Steraloids (Newport, RI).

Polyclonal antibodies (pABs) against Bsep, Mrp2 and Abcg5 have been described previously [1-3]. Hybridomas secreting monoclonal antibodies (mABs) against aminopeptidase N (APN) and dipeptidylpeptidase IV (DPPIV) (CLB4/44) have been described previously [4, 5]. mAB against Mdr1 (C219) was from Alexis Biochemicals (Lausen, Switzerland). Anti-Caveolin-1 pABs, anti-reggie-1/flotillin-2 mABs and anti-reggie-2/flotillin-1 mABs were purchased from BD Transduction Laboratories (Heidelberg, Germany) and anti pan-actin pABs (pan Ab-5) were from Lab Vision (Basel, Switzerland). Secondary antibodies were purchased from GE Healthcare (GE Healthcare Bio-Sciences, Little Chalfont, UK).

DRMs isolation in presence of cholesterol and phosphatidylcholine: Cholesterol and phosphatidylcholine were solubilised in chloroform, dried at 40°C under a mild nitrogen stream and subsequently evaporated for 30 minutes under vacuum. The cholesterol/phosphatidylcholine mixture was then resuspended in 50 mM taurocholate in TNE (150mM NaCl, 1mM EDTA, 20mM Tris-HCl pH 7.4) by sonication and used for BSRM isolation as described in the main text.

SDS-PAGE and immunoblotting: For protein analysis, 50µL of each gradient fraction were used for SDS-PAGE followed by Western blotting as detailed in [2]. Protein distribution in the fraction was evaluated by scanning Western blots films on a Camag Scanner III (Camag, Muttens, Switzerland) at 366nm.

Protein Concentration: Protein concentration was measured using the bicinchoninic acid assay (Interchim, Montluçon France).

Lipids extraction and lipids analysis: Lipids were extracted from cLPMs or fractions as described in [6]. Lipid extraction was performed on 400 µl of each fraction or cLPMs (300 µg total protein) as described in [2], except that extracted lipids were dissolved in 200µl and 500µl trichloromethane for gradient fractions and for cLPMs, respectively. Quantification of cholesterol was performed with a Camag Scanner III (Camag, Muttenz, Switzerland).

Supplementary tables:

Table1: Bile salt composition of bile from male Sprague Dawley rats (molar percent):

CA	CDCA	β MCA	α MCA	DCA	HDCA	UCA	Total BA Concentration mM	<u>PL</u>	<u>C</u>	Ref.
48.4	32.6	12.7	n.a.	6	n.a.	n.a.	n.a.			[7]
45	4.5	37.8	1.8	1.8	6.6	2.3	n.a.			[8]
67	11	13		5	n.a.	4	n.a.			[9]
44.2	9	11.8	7.9	4.6	6.6	1.1	36.3	<u>3.9</u>		[10]
95.2	1.23	n.a.	n.a.	1.63	n.a.	2.59	25.3* 23.14			[11]
78.88	6.87	3.07	trace	5.06	1.15	n.a.	33.5*	<u>6.2</u>	<u>0.73</u>	[12]
								<u>5</u>	<u>0.9</u>	[13]
									<u>0.4</u>	[14]

CA: Cholic acid, CDCA: Chenodeoxycholic acid, MCA: Muricholic acid, DCA: Deoxycholic acid, HDCA: Hyodeoxycholic, UCA: Ursocholic acid, BA: Bile acid, PL: Phospholipids (almost exclusively phosphatidylcholine), C: Cholesterol.

Data are given for taurine conjugated BA except values in bold which correspond to the sum of taurine and glycine conjugated forms.

Content was determined by HPLC, except for data follow by * which were determined by enzymatic assay.

n.a.: not available.

Table 2: CMC values of bile salts and detergents used

Bile salt/Detergent	CMC (mM)		References
	in water	in 0.15M NaCl	
Taurocholate	9 ^a -10	6	[15]
	10	6-9 ^b	[16]
	3-5 ^c	3-4 ^c	[17]
			[18]
Tauroursodeoxycholate	9 ^a -8	2.2	[15]
	8	2.2-2.3 ^b	[16]
		1.6 ^b -1.3-3.8 ^d	[17]
			[19]
Glycocholate	9 ^a -2	11	[15]
	12	11-9 ^b	[16]
	8.1 ^c	6.1 ^c	[17]
			[18]
Taurodeoxycholate	8 ^a -6	2.4	[15]
	6	2.4-3 ^b	[16]
	2.9 ^c	2.0 ^c	[17]
			[18]
Taurodehydrocholate	<250		[17]
CHAPS	6.41 ^c	6.21 ^{d,e} -5.94 ^{d,f}	[20]
Octylglucoside	25		[21]
Muricholic acid		4	[22]
Taurochenodeoxycholate	8 ^a - 7	3	[15]
	7	3-4 ^b	[16]
		1.8 ^b -2.0-2.5 ^d	[17]
			[19]

CMC measured by surface tension except for:

^a Conductimetric data

^b Dye solubilisation

^c Fluorescence measurement

^d Light scattering measurement

^e CMC measured at 0.1M NaCl

^f CMC measured at 0.2M NaCl

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